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Induction of proline-rich tyrosine kinase2 (Pyk2) through C/EBPβ is involved in PMA-induced monocyte differentiation

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Abstract **Proline-rich tyrosine kinase2 (Pyk2) is a cytoplasmic tyrosine kinase related to focal adhesion kinase. Pyk2 expression has been known to be restricted to neuronal and hematopoietic cells and Pyk2 tyrosine phosphorylation and its kinase activity is important for the function of monocytes/macrophages. In NB4 acute promyelocytic leukemia cells, the expression of Pyk2 was increased in parallel with differentiation, and inhibited by PD98059, indicating Pyk2 expression is regulated through MAPK/ERK pathway. Dominant-negative kinase-deficient mutant of Pyk2 reduced the differentiation of NB4 cells in response to phorbol 12-mystate 13-acetate. Transcription factor CCAAT enhancer-binding protein (C/EBP) β was required to induce Pyk2 expression in promoter analysis. These results suggest that Pyk2 is induced and involved in monocyte differentiation and C/EBPβ is a critical regulator of the Pyk2 expression.**

Keywords: Proline-rich tyrosine kinase2 (Pyk2); C/EBPβ; PMA; Monocyte differentiation

1. Introduction

Monocytes and macrophages are cells that serve critical functions in immune defense system, including the phagocytosis of microbial pathogens, the proteolytic processing and presentation of foreign antigens, and the elaboration of a repertoire of cytokines. Circulating monocytes are released from the bone marrow and circulate in the blood for 1–3 days. Following recruitment to tissues, they can differentiate into macrophages, as has been shown in vitro [1]. These processes of cell formation are called monopoiesis. During monopoiesis, monocytes/macrophages undergo several phenotypic changes, such as lamellipodia protrusion, ruffle formation, adhesion, and spreading. Monocyte adhesion to endothelial cells is an essential prerequisite for transmigration leading to their recruitment into tissue and subsequent differentiation into macrophages [2]. The mechanism of formation of adhesion contacts in macrophages, known as podosomes, is not well understood since monocyte/macrophages do not express focal adhesion kinase (FAK) [3].

Proline-rich tyrosine kinase 2 (Pyk2), also known as related adhesion focal tyrosine kinase (RAFTK) or cell adhesion kinase (CAK), is a cytoplasmic tyrosine kinase related to FAK [4]. Pyk2 shows considerable sequence homology and structural similarity to FAK, including consensus motifs in the catalytic domain. Unlike FAK, Pyk2 expression is relatively restricted. Pyk2 is predominantly expressed in the central nervous system and in cells and tissues derived from hematopoietic lineages. In neuronal cells, Pyk2 links various extracellular stimuli that lead to elevation of intracellular calcium concentration, such as activation of the G-protein-coupled receptors, membrane depolarization, with ion channel and mitogen-activated protein kinase functions [5,6]. It has also been demonstrated that Pyk2 plays a role in signaling by lymphocyte antigen receptors, integrin receptors, and chemokine receptors in hematopoietic cells [7,8]. Recently, Pyk2 has been shown to play a role in macrophage morphology and migration in response to lipopolysaccharide and chemokines [9,10]. So it is possible that Pyk2 participates in differentiation of monocytes/macrophages and the level of Pyk2 is regulated during this process. However, little is known about the role of Pyk2 in monocyte differentiation and the regulation of Pyk2 gene expression as yet.

Several transcription factors are known to be involved in monocyte differentiation. The CCAAT enhancer-binding protein (C/EBP) represents a family of transcription factors which has been suggested to play a role in monocyte differentiation [11]. C/EBP is a family of basic region-leucine zipper (bZIP) protein and has C/EBPα, β, γ, δ, ε, and ζ isoforms. These proteins dimerize through their leucine zipper domains and bind to DNA through their adjacent basic regions. C/EBPα,C/EBPβ, C/EBPδ, or C/EBPε can induce granulocytic differentiation in myeloblastic cell lines [12], and C/EBPα, C/EBPβ, or C/EBPδ reported to induce monocytic genes in P388 lymphoblasts [13]. C/EBPβ, in particular, has been shown to be induced upon monocyte differentiation [14,15] and to be a potent activator in the induction of acute phase and inflammatory genes responsive to LPS, IL-1, or IL-6 [16]. C/EBPβ-deficient mice are viable but show defects in innate immunity that result from impaired bactericidal activity of macrophages [17].

In this study, we investigated whether Pyk2 is induced and involved in monocyte differentiation. And we also examined

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Abbreviations: Pyk2, proline-rich tyrosine kinase2; FAK, focal adhesion kinase; PMA, phorbol 12-mystate 13-acetate; C/EBPβ, CCAAT enhancer-binding protein β; FN, fibronectin; MEK/ERK, MAPK/ERK kinase/extracellular signal-regulated kinases; NBT, nitroblue tetrazolium; ChIP, chromatin immunoprecipitation

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the molecular mechanism of Pyk2 induction. Our finding is the first report clarifying Pyk2 is involved in phorbol 12-mystate 13-acetate (PMA)-induced monocytic differentiation.

2. Materials and methods

2.1. Cell culture, differentiation and nitroblue tetrazolium (NBT) reduction assay

Human promyelocytic cell line NB4 (gifted from Dr. Hyung Hoi Kim, Pusan National University) were maintained in RPMI 1640 medium supplemented with 1% penicillin–streptomycin (PS), and 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL). Human cervical carcinoma HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% PS, and 10% FBS. They were maintained in a 37 °C, 5% CO_2, fully humidified incubator, and prepared for experimental procedures when they were in log-phase growth.

To induce monocytic differentiation, NB4 cells were incubated with 20 nM PMA (Sigma, St. Louis, MO) for up to 72 h. The morphological change of NB4 cells to the monocyte/macrophage phenotype was easily detected with inverted microscopy (ZEIZZ). After the treatment of PMA, cells were harvested, and resuspended in PBS containing 2 mg/ml of NBT (Amnesco) and 20 ng/ml of PMA. After incubated at 37 °C for 1 h, incubation was stopped by the addition of cold 2 M HCl. The formazan product was obtained by centrifugation of the sample at $700 \times g$ for 10 min. The supernatant was discarded, and the formazan was dissolved in DMSO. The absorbance was measured at 595 nm.

2.2. Flow cytometry

The expression of the myeloid-monocyte specific antigen CD14 on NB4 cell surface was determined by direct immunofluorescent staining with FITC-conjugated mouse monoclonal antibodies against human CD14 (Beckman-Coulter). FITC-conjugated mouse IgG2a was used as the negative control. The cells were harvested and then suspended in PBS. The FITC-conjugated CD14 or IgG2a antibody was added to cells at room temperature for 30 min, protected from light. Cells were washed with PBS and assayed by flow cytometry (Beckman-Coulter).

2.3. Western blot analysis

Equal amount of whole cell extract was separated by SDS/PAGE (10% gel) and transferred PVDF membranes (Millipore, Dedford, MA). The membranes were incubated with anti-Pyk2, anti-myc, anti-β-tubulin, anti-C/EBPβ (Santa Cruz Biotechnology, CA), or anti-phospho-Pyk2 (Cell Signaling Technology Inc., MA) and then peroxidase conjugated secondary antibody. The protein bands were detected by the enhanced chemiluminescence detection system according to the recommended procedure (Amersham Co, Arlington Height, IL).

2.4. RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using Trizol reagent (Invitrogen) according to the manufacturer's instruction. The cDNA was synthesized from 2 μg of total RNA using AMV-RTase (Promega, Mannheim, Germany) at 42 °C for 1 h. The synthesized cDNA was amplified using Pyk2 and β-actin primers with DYAD thermocycler (MJ Research, Watertown, MA, USA). PCR primers for Pyk2 were 5′-CAAATGCACTGTCCAGACG-3′ (sense) and 5′-TGGGCTTTA-AGTTCTCCTGC-3′ (antisense); for β-actin were 5′-GACTACCTC-ATGAAGATC-3′ (sense) and 5′-GATCCACATCTGCTGGAA-3′ (antisense). PCR reactions were cycled as follows: initial denaturation at 95 °C for 2 min and then 30 cycles for Pyk2 and β-actin at 95 °C for 20 s, 56 °C for 40 s, and 72 °C for 30 s. PCR products were examined by electrophoresis on a 1% agarose gel.

2.5. Cloning of the Pyk2 promoter region

Pyk2 promoter region was amplified by nested-PCR using genomic DNA of human spleen (BioChain, Hayward, CA) as a template. Primary PCR primers were 5′-TTAAGACTCCACACCCCTGG-3′ (sense) and 5′-ATCCCATCACAAACTCCCC-3′ (antisense). The PCR product was nested amplified with sense primer containing the KpnI restriction site (5′-CCCGGTACCTACTGACAGCATCCTTA-CATTCC-3′) and antisense primer containing the SacI restriction site (5′-CCCGAGCTCAAGTAGGTTTCCAGTAACACGG-3′). The amplified DNA fragment −2063/+113 was cloned into the pGL3-Basic luciferase reporter vector (Promega) at KpnI/SacI site (pGL3B/Pyk2-2063). Using this pGL3B/Pyk2-2063 as a template, serial 5′ end deletion constructs were amplified by PCR with the same antisense primer and sense primers containing the KpnI site:

Pyk2-1681-sense (5′-CCCGGTACCAACATGGTGAAACCCTGCC-3′),

Pyk2-1286-sense (5′-CCCGGTACCTTCTCCATGTTAGTTTTGC-CCTG-3′),

Pyk2-479-sense (5′-CCCGGTACCTTCTCCACCAGGTTGTTGAG-G-3′),

Pyk2-321-sense (5′- CCCGGTACCGTAGCAGACGGCGCAGAG-CAAC-3′).

2.6. Transient transfection and luciferase assay

HeLa cells were transfected with plasmids using Polyfect reagent (Qiagen, Valencia, CA) according to the manufacturer's instruction. pSV110-β-galactosidase control plasmid was cotransfected as an internal control for transfection efficiency. After 48 h of transfection, cells were lysed in the cell culture lysis buffer (Promega). Luciferase activities were measured with the luciferase assay substrate (Promega) using Victor 3 luminometer (Perkin–Elmer, Rockville, MD). Transfection efficiency was corrected by β-galactosidase activity. All transfection results represent the mean of three independent experiments.

2.7. Chromatin immunoprecipitation (ChIP) assay

NB4 cells were collected and cross-linked with 1% formaldehyde at 37 °C for 10 min, then were rinsed with ice-cold phosphate-buffered saline twice. Cells were then resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–Cl, pH 8.1, 1 mM PMSF, protease inhibitor cocktail (Roche)) and sonicated nine times for 10 s followed by centrifugation for 10 min. Supernatants were collected and diluted in buffer (1.1% Triton X-100, 0.01% SDS, 1.2 mM EDTA, 167 mM NaCl, 16.7 mM Tris–HCl, pH 8.1) followed by immunoclearing with protein A-agarose and sheared salmon sperm DNA for 30 min at 4 °C. Immunoprecipitation was performed overnight at 4 °C with the C/EBPβ antibody (Santa Cruz Biotechnology). After immunoprecipitation, protein A-agarose with sheared salmon sperm DNA was added and the incubation was continued for another hour. Precipitates were washed sequentially in the following three different washing buffers for 5 min each; a low salt washing buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 150 mM NaCl), a high salt washing buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 500 mM NaCl), and a LiCl washing buffer (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris–HCl, pH 8.1). Immune complexes were washed twice with Tris–EDTA buffer and eluted three times with 1% SDS, 0.1 M $NaHCO_3$. The eluted materials were pooled and heated at 65 °C for at least 6 h to reverse the formaldehyde cross-linking. DNA fragments were purified and reserved for PCR amplification. The primers were used as follow: −479 sense (5′-CCCGGTACCTTCTCCACCAGGTTGTT-GAGG-3′), +113 antisense (5′-CCCGAGCTCAAGTAGGTTTC-CAGTAACACGG-3′).

3. Results and discussion

3.1. The expression of Pyk2 is upregulated during PMA-induced monocytic differentiation of NB4 cells

The human promyelocytic leukemia cell lines NB4 is a useful model for the study of cellular differentiation. NB4 cells can be differentiated toward monocytes/macrophages by PMA [18]. It was assessed the differentiating potential of PMA in NB4 cells using cell morphology, NBT reduction, and CD14 expression.

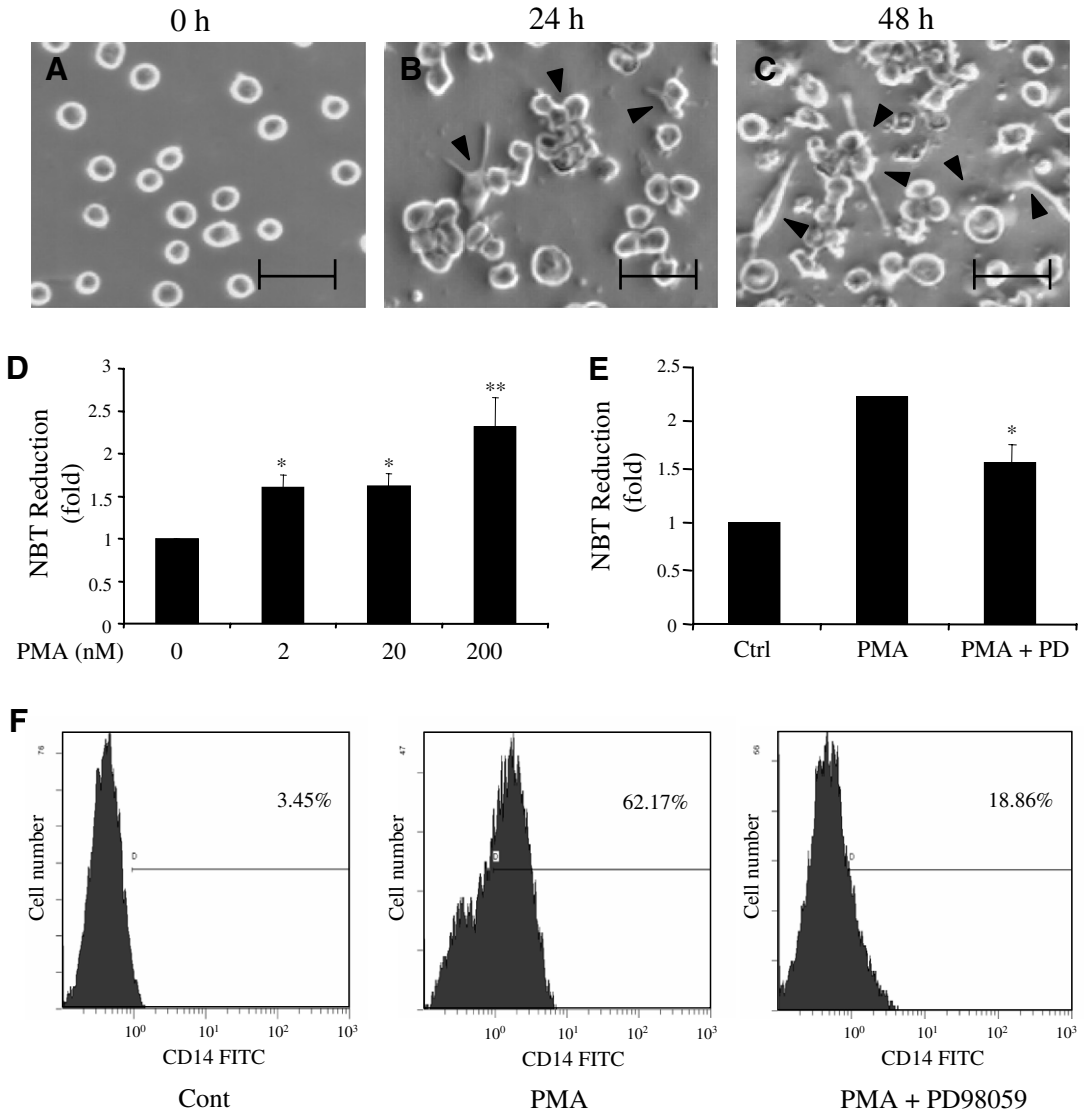

Fig. 1. PMA induces the differentiation of NB4 cells. (A–C) PMA-induced morphological changes of NB4 cells. NB4 cells were incubated with PMA (20 nM) for indicated time. Images of cells in culture at this time point were obtained using an inverted phase contrast microscope, attached to a video camera, and captured using Axio Vision AC image software. PMA induced the aggregation and adherence of NB4 cells (arrowhead). Scale bar is 50 μm. NB4 cells were treated with the indicated concentrations of PMA for 48 h at 37 °C (D) or pretreated with 50 μM of PD98059 for 2 h at 37 °C and then stimulated with 20 nM of PMA for 48 h at 37 °C (E, F) and differentiation was measured by the NBT reduction assay as described in Section 2. Results were presented as the means ± S.E. of four individual experiments performed in duplicate. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control group (D) or $^{*}P < 0.05$ versus PMA alone (E). Cell-surface expression of monocyte differentiation marker CD14 on NB4 cells was analysed by flow cytometry (F) as described in Section 2.

Treatment with 20 nM of PMA-induced cell adhesion and spreading onto culture dish plates, which are the hallmarks of monocyte differentiation (Fig. 1A–C). Some cells clustered and a few cells attached and spread out at 24 h, and more cells were attached and displayed the extended shape at 48 h. PMA-treated NB4 cells revealed the increased NBT reduction in a dose-dependent manner (Fig. 1D). Flow cytometry analysis of the cell surface expression of the monocyte differentiation marker CD14 confirmed that NB4 cells were differentiated by PMA (Fig. 1F).

The adhesion and migration of monocytes/macrophages requires orderly changes in cytoskeletal structures and focal contacts. Recent reports demonstrated that Pyk2 is involved in F-actin organization during maturation of oocyte [19] and regulates differentiation of keratinocytes and neuronal cells [20,21]. Moreover, Pyk2 has been reported to participate in M-CSF signaling of monocytes/macrophages [22]. So it is possible that Pyk2 might involve in the phenotypic changes and differentiation process. To examine whether the level of Pyk2 is changed during monocyte differentiation, NB4 cells were treated with PMA and the expression level of Pyk2 was determined by Western blotting and RT-PCR. PMA increased the protein and mRNA level of Pyk2 in dose- and time-dependent manners (Fig. 2A–D) and the increased expression of Pyk2 was paralleled with the extent of differentiation (Fig. 1A–D). These results suggest that Pyk2 expression is upregulated during PMA-induced monocytic differentiation.

PMA-initiated signals have been known to activate the MAPK cascade including MAPK/ERK kinase/extracellular signal-regulated kinases (MEK/ERK) [23]. To gain insight into

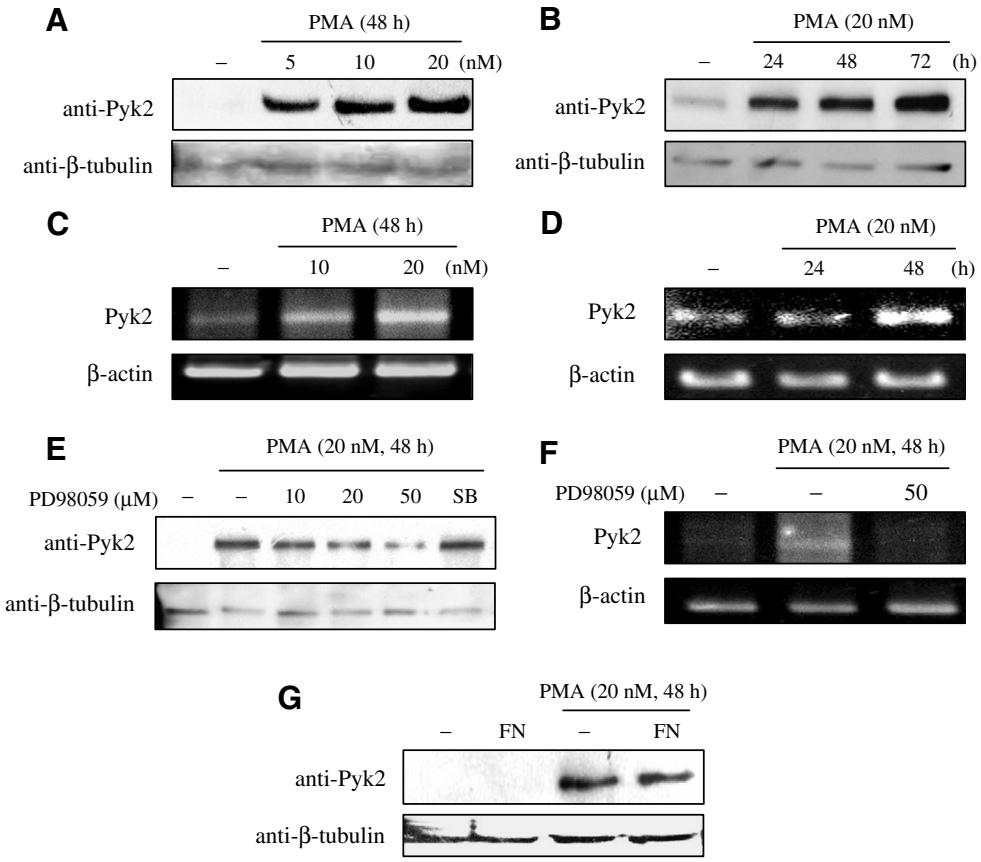

Fig. 2. The expression of Pyk2 was induced by PMA during monocyte differentiation. (A, B) NB4 cells were treated with PMA and equal whole cell lysates were analyzed by immunoblot with anti-Pyk2 antibody. Western blot detection of β-tubulin was estimated protein-loading control for each lane. (C, D) NB4 cells were treated with PMA and RT-PCR was performed using Pyk2- or β-actin-specific primers, as described in Section 2. The β-actin gene expression was analyzed to control for mRNA integrity and loading. (E, F) NB4 cells were pretreated with indicated concentration of PD98059 or 20 μM of SB203580 (SB) and then stimulated with PMA. Western blotting (E) and RT-PCR (F) were performed as described above. (G) NB4 cells were seeded in FN-coated or non-coated plastic culture dishes and treated with PMA. Equal whole cell lysates were analyzed by immunoblot with anti-Pyk2 antibody.

the pathways that regulate the Pyk2 expression in NB4 cells, we examined the effect of the MAPK inhibitor PD98059 (ERK inhibitor), and SB203580 (p38 inhibitor) on the expression of Pyk2. While SB203580 did not block the expression of Pyk2 (Fig. 2E), PD98059 blocked the PMA-induced increase of Pyk2 (Fig. 2E and F). In addition, PD98059 blocked the PMA-induced increase of NBT reduction (Fig. 1E) and cell surface CD14 expression (Fig. 1F). These data suggest that the MEK-ERK cascade is involved in the expression of Pyk2 during PMA-induced monocytic differentiation.

Since fibronectin has been recognized as the key element in promoting cell adhesion and various functions of monocyte [24], we examined the effect of fibronectin (FN) on the expression of Pyk2 during PMA-induced monocytic differentiation. NB4 cells were seeded onto FN-coated or non-coated plastic culture dishes and treated with PMA. FN alone did not induce adhesion and morphological change, and costimulation of FN and PMA had similar effect on differentiation of NB4 cells to PMA alone (data not shown). As shown in Fig. 2G, FN alone did not induce Pyk2 and PMA-induced expression of Pyk2 was not affected by the presence of FN. These results indicate that the induction of Pyk2 by PMA is not dependent on FN.

3.2. Kinase-deficient mutant of Pyk2 suppresses the monocytic differentiation of NB4 cells

In order to verify that Pyk2 is involved in monocyte differentiation, NB4 cells were transfected with myc-tagged wild-type (WT) Pyk2 plasmid or myc-tagged kinase-deficient (KD) K457A-Pyk2 plasmid, containing a lysine (K) to alanine (A) mutation in the ATP-binding site of catalytic domain and then treated with PMA for monocyte differentiation. After 30 h, cell-surface expression of monocyte differentiation marker CD14 on NB4 cells was analyzed by flow cytometry. While cells transfected with WT-Pyk2 expressed slightly more CD14 on their surface in response to PMA, CD14 expression by PMA was significantly reduced in cells transfected with KD-Pyk2 (Fig. 3). This result provides direct evidence that Pyk2 expression plays an important role in monocyte differentiation. To assess the impact of PMA and KD-Pyk2, the level of phospho-Pyk2 (as an indicator of Pyk2 activation) was monitored in plasmid-transfected cells in the absence or presence of PMA (Fig. 3E). WT-Pyk2 or KD-Pyk2 plasmids were expressed in similar extent, while the expression of transfected plasmids was slightly upregulated by PMA (anti-myc in middle panel). PMA induced the phosphorylation of Pyk2 in mock or WT-Pyk2 transfected cells but it failed to phosphorylate

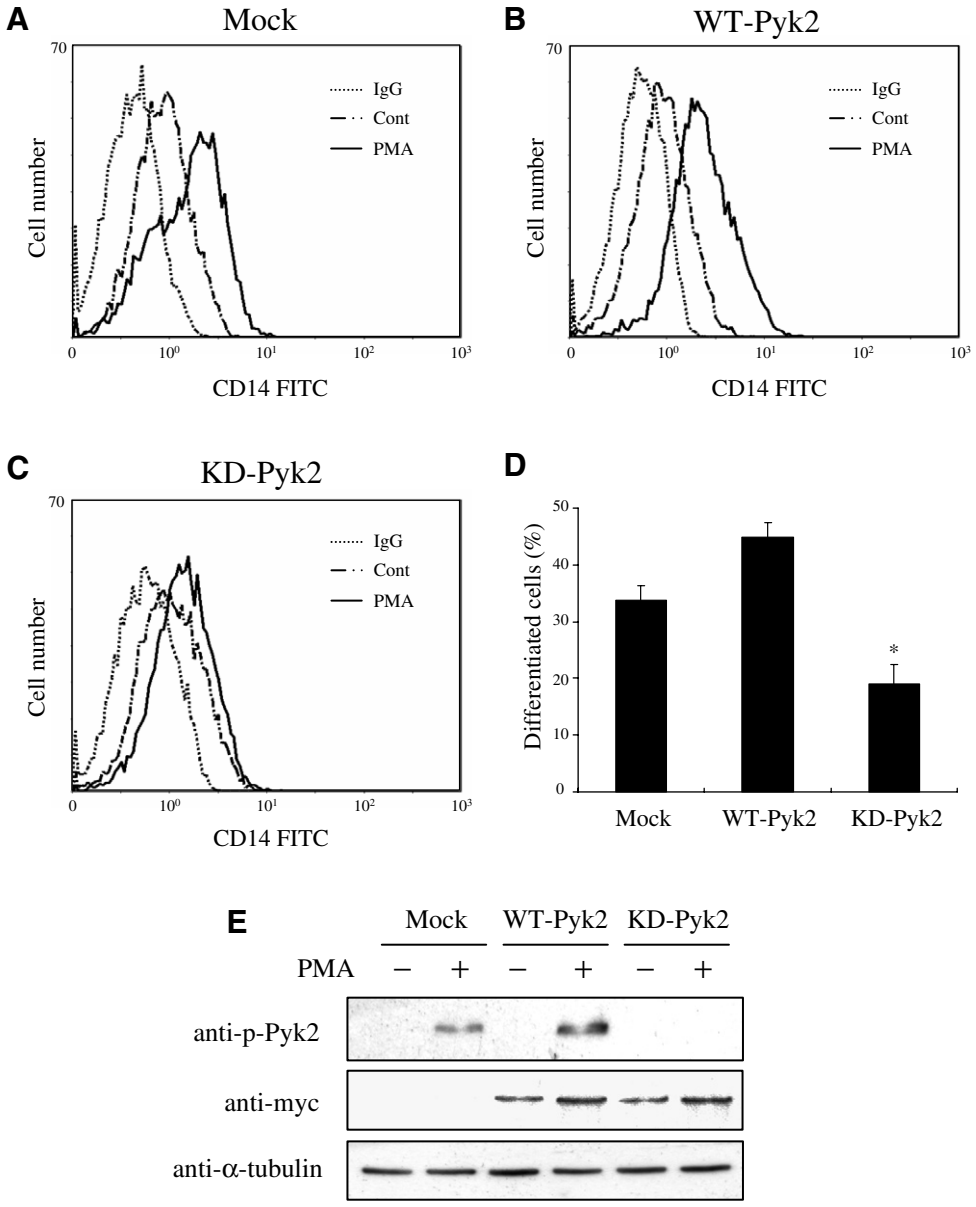

Fig. 3. Kinase-deficient mutant of Pyk2 suppresses the monocytic differentiation of NB4 cells. NB4 cells were transiently transfected with pcDNA3.1 (mock, A), full length Pyk2 (WT-Pyk2, B) or kinase-deficient Pyk2 (KD-Pyk2, C) plasmid using FuGENE HD transfection reagent (Roche, IN). After 24 h, differentiation was induced by 20 nM of PMA for 30 h. Cell-surface expression of monocyte differentiation marker CD14 on NB4 cells was analyzed by flow cytometry as described in Section 2. Dot-line (···) indicates experiment with FITC-conjugated mouse IgG2a. (D) Percentage of differentiated cells was calculated with the difference between the percentage of CD14-positive control cells and that of CD14-positive PMA-treated cells of three individual experiments. $^{*}P < 0.05$ versus mock. (E) Equal whole cell lysates were analyzed by immunoblot with anti-myc or anti-phospho-Pyk2 antibody.

KD-Pyk2. This result indicates that PMA activates Pyk2 and KD-Pyk2 acts as a dominant-negative mutant.

As NB4 cells differentiate into monocytes, morphological changes such as adhesion and spreading are accompanied, which requires cytoskeleton rearrangement. Pyk2 is present at lamellipodia of monocytes [25] and studies of cells derived from Pyk2-deficient mice indicate that Pyk2 is important for the contractile activity of lamellipodia in migrating macrophages [10]. And Pyk2 has been reported to be involved in FcγR-mediated phagocytosis [26]. So Pyk2 might be required in differentiation of NB4 cells and increased Pyk2 could play an important role in spreading and motility as well as monocyte/macrophage functions such as phagocytosis.

3.3. C/EBPβ regulates the transactivation of Pyk2 promoter

To further understand the mechanism of Pyk2 expression at the level of gene transcription, we cloned and studied the promoter region of Pyk2 gene. Database analysis of the 5′-flanking region for known consensus sequences in Pyk2 promoter indicates the presence of binding sites for C/EBPβ. In order to elucidate whether C/EBPβ increases Pyk2 expression, HeLa cells were transfected with the indicated amounts of the expression vectors encoding C/EBPβ along with the human Pyk2 promoter luciferase reporter (Pyk2-2063-Luc). The results shown in Fig. 4A showed that C/EBPβ significantly increased the transactivation of Pyk2 promoter. Since it has been known that a naturally occurring isoform of C/EBPβ, LIP (liver-enriched

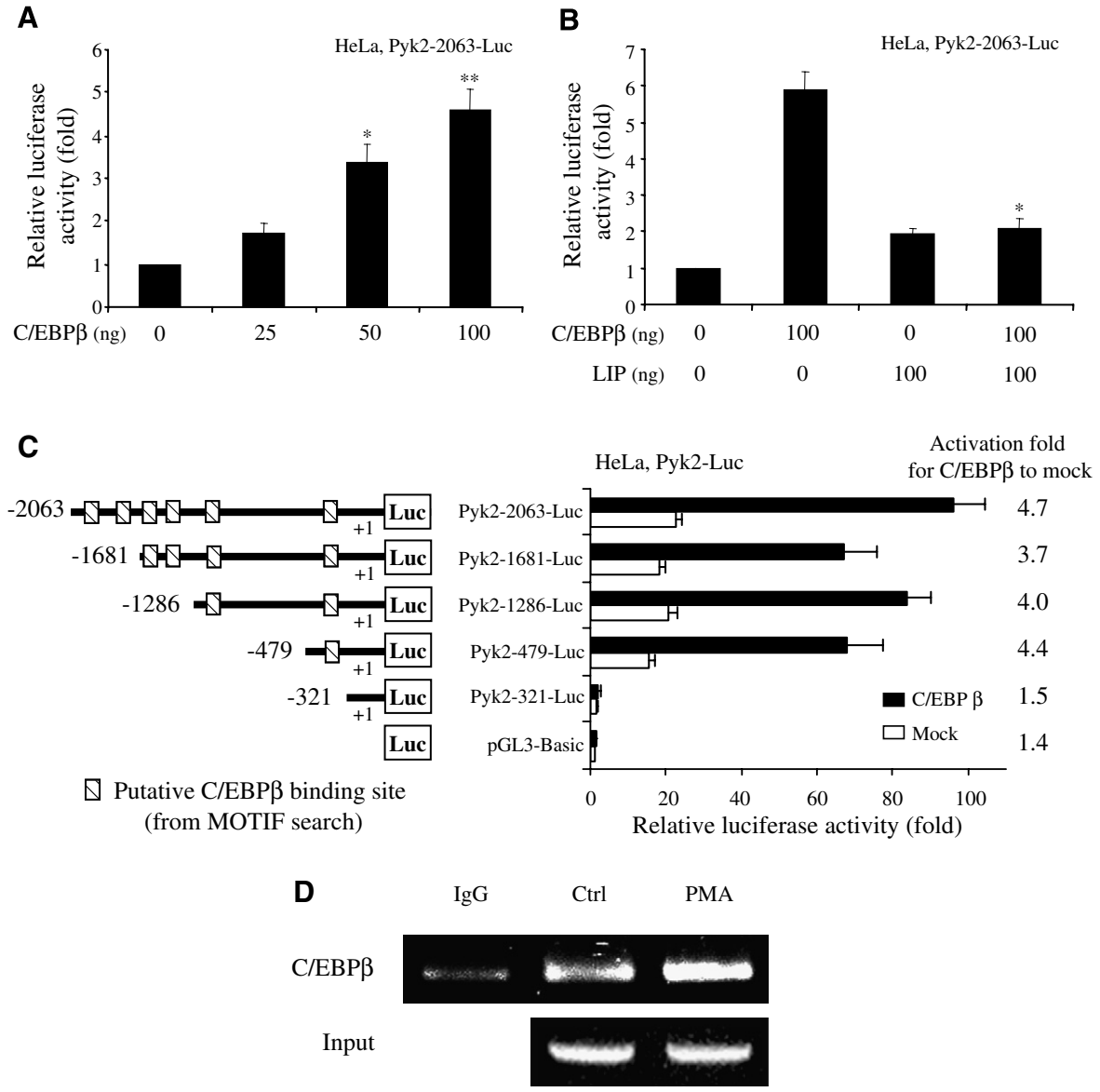

Fig. 4. Transactivation of the Pyk2 promoter by C/EBPβ. (A) HeLa cells were cotransfected with Pyk2-2063-Luc and vector expressing C/EBPβ. Luciferase assay was performed as described in Section 2. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus no C/EBPβ (mock). (B) HeLa cells were cotransfected with Pyk2-2063-Luc and vector(s) expressing C/EBPβ and/or LIP, and luciferase assay was performed as described in Section 2. $^{*}P < 0.05$ versus C/EBPβ alone. (C) Deletion analysis of the 5′-flanking region of Pyk2 promoter. HeLa cells were transfected with the indicated deletion constructs and vector expressing C/EBPβ. Luciferase assay was performed as described in Section 2. (D) Chromatin immunoprecipitation (ChIP) assay of C/EBPβ. NB4 cells were treated with 20 nM of PMA for 48 h at 37 °C. Following formaldehyde cross-linking, purified chromatin was immunoprecipitated with normal mouse IgG or anti-C/EBPβ antibody. DNA isolated from immunoprecipitated material was amplified by PCR with primers for from −479 to +113 regions. Input shows the starting chromatin extracts.

inhibitory protein), exhibits a dominant-negative effect on a C/EBPβ-dependent promoter-reporter due to a lack of the transactivation domain [27], the effect of expression vectors encoding LIP was examined through cotransfection with C/EBPβ-expression vectors. LIP overexpression inhibited C/EBPβ-induced transactivation of the Pyk2 promoter activity (Fig. 4B). These data indicate that C/EBPβ activates the Pyk2 promoter and the transactivation domain of C/EBPβ is essential for its activation of the Pyk2 promoter.

Next, we identified the C/EBPβ responsive region of Pyk2 promoter. It was found that there are six potential C/EBPβ binding sites within 2.063 kb Pyk2 promoter from database search (MOTIF Search, Fig. 4C). To identify the promoter region responsible for C/EBPβ binding to Pyk2 promoter, a series of sequential deleted construct of the Pyk2 promoter region was generated and subjected in reporter assay with cotransfection of C/EBPβ expression plasmid into HeLa cells. As shown in Fig. 4C, the activity of Pyk2-479-Luc promoter construct was well maintained and similar to that of other promoter constructs, while Pyk2-321-Luc promoter construct (not including putative C/EBPβ binding site) was not maintained and not induced by C/EBPβ. Based on this result, Pyk2 promoter region (−479 to −321) including just one putative C/EBPβ binding site was regarded as a C/EBPβ responsive site

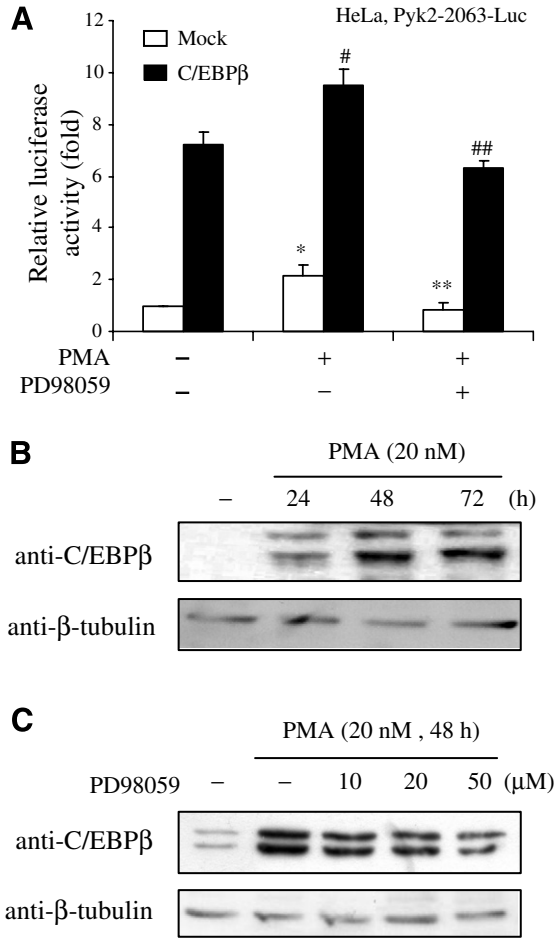

Fig. 5. PMA induces the expression of Pyk2 through MEK/ERK-dependent upregulation of C/EBPβ. (A) HeLa cells were cotransfected with Pyk2-2063-Luc and vector expressing C/EBPβ. Twenty-four hours after transfection, cells were pretreated with 20 μM of PD98059 for 2 h at 37 °C and then stimulated with 20 nM of PMA for 24 h at 37 °C. Luciferase assay was performed as described in Section 2. $^{*,\#}P < 0.05$ versus control (no PMA); $^{**,\#\#}P < 0.05$ versus PMA alone (* and **, mock; # and ##, C/EBPβ). (B, C) NB4 cells were treated with PMA (B) or pretreated with indicated concentration of PD98059 and stimulated with PMA as described in Fig. 2E (C). Equal whole cell lysates were analyzed by immunoblot with anti-C/EBPβ antibody. Western blot detection of β-tubulin was estimated protein-loading control for each lane.

in the transactivation assay through luciferase reporter expression.

To confirm that C/EBPβ could directly bind to the Pyk2 promoter *in vivo* and to further define the C/EBPβ response elements in the promoter, chromatin immunoprecipitation (ChIP) assay was carried out, which detects specific genomic DNA sequences that are associated with a particular transcription factor in intact cells. NB4 cells were treated with 20 nM of PMA for 48 h at 37 °C. After treatment, proteins were cross-linked to genomic DNA and immumoprecipitation with normal mouse IgG, or polyclonal antibody to C/EBPβ was performed. And then PCR was conducted with primers designed to amplify a 609-bp fragment (from −479 to +113). As shown in Fig. 4D, the binding of C/EBPβ to this site of Pyk2 promoter was increased by PMA-treatment. These results suggest that C/EBPβ is responsible to bind and transactivate the Pyk2 promoter.

3.4. PMA induces the expression of Pyk2 through MEK/ERK-dependent upregulation of C/EBPβ

In order to elucidate whether C/EBPβ increases the PMA-induced Pyk2 expression, HeLa cells were transfected with the expression vectors encoding C/EBPβ along with the human Pyk2 promoter Pyk2-2063-Luc and treated with PMA. As predicted, PMA provoked the transactivation of the Pyk2 promoter in HeLa cells and C/EBPβ increased PMA-induced transactivation of Pyk2 promoter (Fig. 5A) indicating that C/EBPβ and PMA act cooperatively to induce transactivation of the Pyk2 promoter. To verify that the expression of C/EBPβ is affected by PMA in NB4 cells, NB4 cells were treated with 20 nM of PMA for the indicated times at 37 °C. PMA increased the level of total cellular C/EBPβ (Fig. 5B) and the expression of C/EBPβ is paralleled with increase in Pyk2 (Fig. 2B). Because PD98059 blocked both the transcription and expression of Pyk2 (Fig. 2E and F), it was investigated the effect of PD98059 on the Pyk2 promoter activity. PD98059 suppressed Pyk2 transactivation by C/EBPβ (Fig. 5A) and PMA-induced expression of C/EBPβ (Fig. 5C). These results suggest that C/EBPβ plays a role as an activator of the transcription of Pyk2 during PMA-induced monocytic differentiation and PMA induces the expression of Pyk2 through MEK/ERK-dependent upregulation of C/EBPβ.

C/EBPα and C/EBPβ have been shown to play an important role in tissue-specific and differentiation-dependent IL-10 transcription [14]. It has been reported that C/EBPβ is a major transcription factor in retinoic acid-induced differentiation of NB4 acute promyelocytic leukemia cells [12] and is required for 1,25-dihydroxyvitamin D3-induced monocytic differentiation of HL60 cells [15]. And C/EBP levels are low in immature myeloid cells, increase after stimulation with LPS, IL-6, and IL-1, and are highest in macrophages and neutrophils [11]. Moreover, PMA, acting through the protein kinase C pathway, has been reported to increase the site-specific phosphorylation and activity of C/EBPβ [28]. Therefore, PMA could not only induce C/EBPβ level but also increase the activity of C/EBPβ through MEK/ERK pathway. These increased and activated C/EBPβ could bind to the promoter of Pyk2 gene and provoke the expression of Pyk2, which might force promyelocytes to differentiate into monocytes/macrophages.

In conclusion, our study shows for the first time that up-regulation of Pyk2 gene is functionally important for monocytic differentiation by PMA and transcription factor C/EBPβ is a critical regulator of the Pyk2 induction. This study further suggests that the transactivational activity of C/EBPβ, the expression of Pyk2 gene and monocyte differentiation is regulated by MEK/ERK signaling pathway. Our finding provides insight into the molecular mechanism of monocytic differentiation in promyelocytic leukemia cells.

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